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(54) Title: DELTA-LIKE GENE EXPRESSED IN NEUROENDOCRINE TUMORS (57) Abstract A polynucleotide molecule <i>dlk</i> is expressed in neuroendocrine tumors, including small cell lung carcinoma. A Dlk polypeptide encoded by <i>dlk</i> polynucleotide molecule can be used in detecting the existence of a primary or secondary neuroendocrine tumor. Monoclonal antibodies are produced against Dlk which are useful for detection and therapy of a neuroendocrine tumor.		

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DELTA-LIKE GENE EXPRESSED IN NEUROENDOCRINE TUMORS

Background of the Invention

The expression of genes during the development of a pluripotent or progenitor cell into a differentiated, mature cell can provide a context for the study of tumorigenic cells whose origin is derived from such progenitor cells. In certain hematopoietic or epithelial tumors, malignant gene expression correlates substantially with the expression observed during normal development of the tissue from which the tumor originates, Gordon et al., *J. Cell Biol.* 108: 1187 (1989); Godal et al., *Adv. Cancer Res.* 36: 211 (1982). In fact, many biological activities of progenitor cells, including cellular migration and tissue remodeling, resemble pathological activities of cancer cells, such as metastases and tumor invasion.

Neuroblastoma, a tumor of the adrenal gland which afflicts persons during early childhood, is another system in which tumor biology correlates with that of normal differentiation and morphogenesis of its progenitor cells (neuroblast). Neuroblastoma is an embryonal tumor that exhibits both undifferentiated and differentiated histopathology. The development of neuroblastoma tumors mimics stages identifiable during histogenesis of its tissue of origin, the adrenal medulla. Cooper et al., *Cell Growth and Diff.* 1: 149 (1989).

During the development of human adrenal medulla neuroblasts into mature chromaffin cells, four individual genes are expressed in a sequential pattern. Once a neuroblast is induced to differentiate along a neuroendocrine pathway, the progressive stages of chromaffin maturation are marked by a temporal expression of genes denoted TH, CGA, pG2 and B2M (Cooper, *supra.* at page 153). Cooper found that the pattern of gene expression of these four markers in neuroblastoma cells mimics that of normal adrenal neuroblast arrested during three different stages of development.

One of these marker genes, pG2, was identified first in pheochromocytoma, a tumor of the adult adrenal medulla (Helman

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et al., *PNAS USA* 84: 2336 (1987)). Helman reported that pG2 also is highly expressed normal human adrenal cells.

Helman isolated a full-length cDNA from a human adrenal cDNA library, and identified a corresponding pG2 protein
5 containing 286 amino acids, having a predicted molecular weight of 30.6 kilodaltons (kDa) (Helman et al., *Nucleic Acids Res.* 18(3): 685 (1990)).

A gene having developmentally-regulated expression, paralleling that of pG2, would be useful for detecting
10 pheochromocytoma or neuroblastoma by genetic methods, especially since pG2 expression is restricted to the adrenal gland in non-malignant tissue.

Summary of the Invention

It is therefore an object of the present invention to
15 provide a newly-isolated polynucleotide molecule, *dlk*, which can be employed in genetic assays to provide a method for detection of a primary or secondary pheochromocytoma or neuroblastoma, or identification of a stage of these tumors.

It is also an object of the present invention to provide
20 a method for detecting primary or secondary small cell lung carcinoma (hereafter, SCLC) or for staging tumor progression of SCLC, which employs *dlk* polynucleotide molecules in genetic assays.

It is a further object to provide a polynucleotide
25 molecule, designated *dlk*, which encodes a corresponding Dlk polypeptide. Dlk polypeptides are useful for generating monoclonal or polyclonal antibodies having specificity for an epitope of the Dlk polypeptide.

Dlk-specific antibodies, and in particular, labeled
30 monoclonal Dlk-specific antibodies, are useful for detection of primary or secondary neuroendocrine tumors. According to the present invention, Dlk-specific monoclonal antibodies conjugated to a toxin are useful for treatment of primary or secondary neuroendocrine tumors, as well.

35 In accomplishing these and other objects of the invention, there has been provided, in accordance with one

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aspect of the present invention, an isolated polynucleotide molecule comprising a DNA sequence encoding a Dlk polypeptide.

An object of the present invention is to provide an isolated Dlk polypeptide consisting essentially of the amino acid sequence shown in Figure 1B, or in Figure 1A.

Another object of the present invention is to provide an isolated polynucleotide molecule which encodes a human or mouse Dlk polypeptide consisting essentially of the amino acid sequence shown in Figures 1B, or 1A, respectively.

A further object of the invention is to provide a method for detecting a tumor which expresses dlk, including the steps of contacting RNA from a sample suspected of being tumorigenic with a dlk polynucleotide molecule, under conditions permissive of hybridization between dlk polynucleotide molecule and the sample, and detecting hybridization between the polynucleotide molecule and sample.

Yet another object of the invention is to provide a method for detecting a small cell lung carcinoma, including the steps of contacting RNA from a sample of bronchial epithelial cells suspected of being tumorigenic with dlk polynucleotide molecules, under conditions permissive of hybridization between dlk polynucleotide molecules and the sample, and detecting hybridization between the polynucleotide molecules and sample.

Brief Description of the Drawings

Figure 1 shows an alignment of mouse (Figure 1A) and human (Figure 1B) Dlk amino acid sequences. Identical amino acids are shown by the character (|). Similar amino acids are indicated by (^) and classified into the following groups: A, S & T; D & E; N & Q; R & K; I, L, M & V; and F, Y & W. Potential biologically significant sites, found in the database PROSITE (accessible commercially through Intelligenetics Inc. (Mountain View, CA)), are indicated by numbers: 1. N-glycosylation site; 2. Protein Kinase C phosphorylation site; 3. N-myristylation site; 4. Aspartic acid and asparagine hydroxylation site. Potential sites of cleavage in the signal peptide are indicated by (*).

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Figure 2 shows the human *dlk* DNA sequence.

Figure 3 shows the mouse *dlk* DNA sequence.

Figure 4 shows an alignment of a consensus sequence of *dlk* EGF-like repeats with EGF-repeats found in several invertebrate homeotic genes. As described in Example 4, a *dlk* EGF-like repeat consensus sequence was obtained by alignment of 12 EGF-like repeats of *dlk* from both human and mouse. This consensus sequence then was aligned with the consensus sequences of several invertebrate homeotic genes (similarly obtained) and mouse EGF.

Detailed Description of the Preferred Embodiments

A human polynucleotide molecule, *dlk*, and a corresponding human polypeptide, Dlk, encoded by *dlk*, were discovered, isolated and characterized. The human *dlk* polynucleotide molecule was found to be expressed in pheochromocytoma, neuroblastoma, and SCLC tumors.

Dlk protein is about 383 amino acids in length and has a molecular weight of about 42 kDa. In addition to human *dlk*, other polynucleotide molecules belonging to the *dlk* family are provided according to the invention, including murine *dlk* (Figure 3) and a human variant-*dlk*, isolated from placenta as described herein.

According to the present invention, isolated polynucleotide molecules or fragments thereof belonging to the *dlk* family are useful in the detection of SCLC and neuroendocrine cancers. The expression patterns of *dlk* can be exploited both (1) to detect primary or secondary tumor cells by the presence of *dlk* and (2) to diagnose the stage of a tumor that expresses *dlk*, by measuring the level of *dlk* expression.

Dlk is a transmembrane protein having an expression pattern, in normal non-fetal tissues, which is restricted to the adrenal gland. As a consequence, Dlk is a readily accessible target for antibody imaging or therapy of SCLC, pheochromocytoma and neuroblastoma tumors. According to the present invention, antibodies having specificity for the Dlk

protein are made and employed to detect or treat cells which produce the Dlk protein.

Human *dlk* cDNA comprises a polynucleotide molecule having the sequence shown in Figure 2, as determined by nucleotide sequence analysis. The open reading frame, nucleotides 174(ATG) - 1322(TAA), is 1149 nucleotides long. The mouse *dlk* polynucleotide molecule comprises a DNA sequence having an open reading frame, nucleotides 134(ATG) - 1288(TAA), of 1155 nucleotides, as shown in Figure 3. The murine Dlk protein is about 385 amino acids and has a molecular weight of about 42 kDa.

According to the present invention, a variant of human Dlk is identified in which an amino acid is deleted. A cDNA encoding "variant-Dlk," in which amino acid number 347 of the amino acid sequence shown in Figure 1(B) is deleted, was isolated from a total human placental cDNA library. Amino acid position 347 occurs in an intracellular domain of the protein. The placental library containing variant-dlk also contained substantial amounts of the non-variant form, that is, *dlk* polynucleotide molecule shown in Figure 1B.

The *dlk* polynucleotide molecule was identified by examination of cDNA expression products of human SCLC (hSCLC) lines which were responsive to stimulation with the ligand, gastrin-releasing peptide (GRP), a neuropeptide implicated in the release of gastrin through its interaction with a G-protein-coupled receptor, GRP receptor. GRP (peptide) is a mitogen for both normal lung epithelial cells and SCLC cells, and for murine Swiss 3T3 fibroblasts.

GRP-responsive hSCLC lines were compared with murine fibroblast cell lines that were differentially responsive to GRP. This approach, as detailed in Example 1, yielded a partial length cDNA molecule that hybridized a 1.6 Kb mRNA expressed both in responsive fibroblasts and responsive SCLC lines. A commercially available Swiss 3T3 fibroblast cDNA library was screened with the partial length cDNA, which yielded several clones having 1.6 Kb inserts, which then were sequenced.

A computer search of the databases "Swissprot" and "NBRF Protein," described by Devereux et al., *Nuc. Acids Res.* 12(1): 387 (1984), indicated a high degree of homology between Dlk and proteins encoded by several homeotic genes, identified in Example 3. Homeotic genes are development-controlling regulatory genes that assign spatial identity to groups of cells with respect to their morphogenic fates. In segmented organisms, for example, homeotic genes are required for the proper morphogenesis of a distinct region (such as a leg, or antennae) and act by controlling the activities of other genes during development. The Dlk protein of the present invention exhibited highest homology with the protein Delta, a neurogenic locus involved in normal neural differentiation in *Drosophila*. Thus, the present protein was designated "Dlk" for being "delta-like."

Mouse and human Dlk protein sequences share 86.2% identity as well as many potential sites of biological importance, including 6 epidermal growth factor (EGF)-like repeats, a transmembrane domain, and a signal peptide domain at the amino terminus. Based upon these structural features, *dlk* appears to be a new member of the family of EGF-like neurogenic genes of *Drosophila*, which are involved in developmental decisions of the embryonal ectoderm to differentiate into epidermal or neuronal cells.

The expression pattern of *dlk* and its sequence homology with homeotic proteins support the notion that *dlk* functions in the differentiation pathway for cells of the chromaffin lineage. As detailed in Example 2, *dlk* is expressed in primary and secondary pheochromocytoma and neuroblastoma cells, and in normal (nonhistopathological) human adrenal medulla and placental cells. According to the present invention, SCLC and neuroblastoma are the only tumors known to express *dlk* as a function of differentiation.

An isolated *dlk*, *dlk*-variant, and murine *dlk* polynucleotide and protein products are employed in diagnostic methods (described further below) and are made according to the following description. Hereafter, the techniques and

applications described for *dlk* polynucleotide molecule (DNA, RNA) and Dlk protein are intended to be useful for DNA, RNA and protein of murine *dlk*, and of variant-*dlk*, as well.

5 A Dlk polypeptide, according to the present invention, is produced by recombinant DNA techniques, such as those described by Maniatis et al., MOLECULAR CLONING - A LABORATORY MANUAL, Cold Spring Harbor Laboratory (1982). Methods specifically applicable to cloning the *dlk* polynucleotide molecule are described in Example 1.

10 The *dlk* polynucleotide molecule of Figure 1B can be cloned into suitable expression vectors and expressed in prokaryotic, insect or eukaryotic expression systems, including *Baculovirus* or *E. coli* (Boehringer Mannheim). Using conventional techniques, therefore, a polynucleotide sequence encoding a Dlk protein, can be obtained as a cDNA from mRNA
15 from a commercial adrenal medulla or Swiss 3T3 fibroblast library, or from SCLC, neuroblastoma or pheochromocytoma cell lines. The mRNA can be converted to double-stranded DNA using cDNA cloning techniques well-known to the art, including PCR-based techniques. Linkers or tails may be added to the ends
20 of the double-stranded DNA to provide convenient restriction sites. After restriction enzyme digestion, the DNA may be introduced into a cloning vector, such as a plasmid, which has been digested with a restriction enzyme that generates appropriately compatible ends. A suitable plasmid vector in
25 this context is pGEX- λ (Pharmacia). Following ligation, by means of standard techniques, the DNA is introduced into a cell, where its expression produces the desired protein.

Alternatively, a Dlk polypeptide is produced using a
30 commercially available *in vitro* translation kit from NEN (Boston, MA), as detailed in Example 1. This kit employs a translation system (including ribosomes, polymerases, amino acids, etc.) derived from rabbit reticulocyte lysates.

The term "isolated," with reference to the *dlk*
35 polynucleotide molecule indicates that such a molecule is free of the proteins with which it is normally associated, such as histones. An isolated form of the *dlk* is substantially free

of other DNA that does not function to regulate, promote, enhance or otherwise modulate its expression.

The term "isolated," with reference to Dlk protein, connotes a polypeptide that is free of other proteins with which it is normally associated.

An isolated *dlk* polynucleotide molecule is useful for detecting primary SCLC and the metastatic spread of SCLC and other neuroendocrine cancers. More specifically, the present invention provides a method of tumor detection which includes the steps of contacting a sample suspected of containing a tumor with a *dlk* polynucleotide molecule, and detecting expression of *dlk* polynucleotide products (DNA, RNA, mRNA) in non-adrenal cells. Detection of a *dlk* polynucleotide product identifies the cells as metastatic cells (secondary tumor) of neuroblastoma, pheochromocytoma or SCLC, or as a primary tumor of SCLC.

The ability to detect *dlk*-expressing cells is useful for both tumor detection and tumor identification. After detecting *dlk*-expression, a tumor type is determined by detecting either a tumor-specific marker, tumor-specific morphology, or by presentation by the patient of a clinical pathology that is distinctly associated with any of the tumors selected from the group including neuroblastoma, pheochromocytoma or SCLC. For example, information such as the identification of a cellular marker, histological feature or disease symptom which is specific to one of the tumors of neuroblastoma, pheochromocytoma or SCLC, is recognized.

If *dlk* expression is detected in cells of a sample taken from bronchial epithelial tissue or tissue removed from the lung, the observation identifies a primary SCLC. It is preferred that a second step of confirming the origin of the detected *dlk*-expressing tumor cells as SCLC be performed by detection of a marker, histological feature, or presentation of a distinctive symptom associated with this tumor. For example the histology of an "oat cell" commonly associated with SCLC is detected to confirm the presence of SCLC.

The expression of *dlk* is detected by hybridization with a *dlk* polynucleotide probe. This method includes the steps of contacting a suspected tumor sample with a *dlk* polynucleotide molecule, and detecting the hybridization between the polynucleotide molecule and sample. A positive hybridization signal indicates that the sample is of tumor origin.

The polynucleotide molecule or "*dlk* probe" used to detect *dlk* expression is a labeled fragment of *dlk*, or preferably a full-length *dlk* DNA molecule which will hybridize to mRNA or DNA from normal adrenal and neuroendocrine tumor cells. Probes complementary to *dlk* are prepared by conventional methods, and are preferably allowed to hybridize to mRNA or DNA, using conventional *in situ* hybridization techniques. Unhybridized probe is removed by nuclease digestion.

In situ hybridization techniques which are known in the art may employ the use of fluorescent labels and radiolabels which can be easily quantitated by fluorescence microscopy or autoradiography, respectively. Generally, fluorescent labels will be preferred. Another labelling technique may employ enzymatic tags which generate readily quantifiable colorimetric or chemiluminescent signals. The hybridization intensity detected in their procedures reflects the amount of *dlk* within the biological sample.

The *dlk* polynucleotide of the invention can be employed as a probe in RNA ("Northern") blotting procedures. According to this method, RNA is first isolated from tissue by any of a number of standard procedures (Lehrach, H., *Biochemistry*, 16: 4743 (1975)). The RNA sample is then subjected to denaturing gel electrophoresis and is transferred to a nitrocellulose membrane or other solid support matrix. The *dlk* mRNA can be detected by hybridization of radioactively or non-radioactively labelled *dlk*, or *dlk* fragments, preferably under high stringency conditions as would be familiar to one of ordinary skill in the art. The amount of hybridization can be quantified by densitometry.

In yet another embodiment of the present invention, the polymerase chain reaction ("PCR") is used to detect *dlk* DNA or

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mRNA in a sample. To perform PCR, a pair of *dlk* sequence specific polynucleotide primers is employed, which hybridize to opposite strands of the *dlk* gene at offset positions on the double helix. Such primers, taken from the *dlk* polynucleotide sequences provided in accordance with the invention, represent fragments which preferably are unique to *dlk*, e.g., sequences having low homology with other proteins than Dlk. Two exemplary *dlk*-specific primer sequences useful in this context include the following sequences, which encode a portion of the intracellular region of Dlk:

5'-CAA GCC CGA GTT CAC AGG TC-3'

5'-TCG GGG AAG ATG TTG AC-3'.

Other such primer pairs can be selected and utilized, as well.

The primers provide initiation points for DNA synthesis. In the presence of DNA polymerase, the four deoxynucleotide triphosphates ("dNTPs") and other necessary cofactors, all of which are well known to the art, new DNA strands are synthesized complementary to the templates which hybridized with the primers. Several rounds of synthesis are carried out, with allowance for denaturation of the double stranded products between rounds. Preferably, a thermal stable DNA polymerase is used so that it is not necessary to add enzyme anew for each round of synthesis.

The PCR produces a double stranded DNA amplification product which has the same sequence as the original stretch of the *dlk* DNA defined by the ends of the primer pair sequences. The amount of PCR product indicates the amount of *dlk* DNA or *dlk* mRNA in the sample. The product can be detected by a variety of methods well-known in the art. The PCR products can be resolved by agarose or polyacrylamide electrophoresis and detected by fluorescence staining, such as ethidium bromide. Alternatively, one of the dNTPs may be labelled and the PCR products may be determined by measuring incorporation of the labelled dNTP. A variety of other methods for resolving, detecting and quantitating PCR products are well-known to those of ordinary skill in the art.

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The PCR may be made specific for either *dlk* DNA or *dlk* mRNA. For instance, RNase or DNase may be used to remove one template or the other from the sample, and the use of primers that distinguish between the gene and the message, for example, a primer that hybridizes to a sequence in the untranscribed region of the promoter will be specific for the *dlk* gene, and not for the *dlk* mRNA.

Other techniques suitable to the claimed methods are readily apparent to the skilled artisan and can include Nuclease Protection Assays, ELISA and Western blotting. Several assay techniques which are based upon immunological reactions between antigens and antibodies are contemplated by the invention. In particular, assays which use antibodies having specificity for Dlk protein are useful to detect cells which produce Dlk protein.

Antibodies having specificity for Dlk-expressing cells are obtained by immunizing an animal with Dlk protein. In this context, the term "antibody" encompasses both monoclonal and polyclonal antibodies. such an antibody can belong to any antibody class (IgG, IgM, IgA, etc.). According to the present invention, an entire Dlk polypeptide is injected into an animal for the purpose of obtaining polyclonal antibodies, or for obtaining lymphocytes or spleen cells for production of monoclonal antibodies.

The general techniques of monoclonal antibody (MAb) production, such as those described by Kohler and Milstein, *Nature* 256:495 (1975), are applied to produce a monoclonal antibody having specificity for Dlk protein. This procedure includes the steps of isolating lymphocytes of an animal which has been sensitized or injected with Dlk polypeptide, fusing them with a myeloma partner to produce hybridomas, then screening the hybridomas for production of "anti-Dlk antibodies" which exhibit binding specificity for a Dlk polypeptide.

The term "Antibody" also encompasses fragments, like FAB and $F(Ab^1)_2$, of anti-Dlk antibodies, and conjugates of such fragments, and so-called "antigen binding proteins" (single-

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chain antibodies) which are based on anti-Dlk antibodies, in accordance, for example, with U.S. patent No. 4,704,692, the contents of which are hereby incorporated by reference. Alternatively, MAbs or fragments thereof can be produced via
5 the expression of genes which encode variable regions of such an MAb in host cells like *E. coli*, see, e.g., Ward et al., *Nature* 341: 544-546 (1989), or transfected murine myeloma cells. See Verhoyen et al., *BioAssays* 8: 74 (1988); Gillies et al., *Biotechnol.* 7: 799-804 (1989); Nakatani et al.,
10 *Biotechnol.* 7: 805-10 (1989).

Assays in which the above antibodies are employed can include enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays, immunoelectrophoresis, and the like. Also useful diagnostically are immunohistochemical techniques which
15 employ monoclonal antibodies of known reactivity.

In accordance with this aspect of the present invention, a sample is obtained from a person to detect: (1) a small cell lung carcinoma, by removing a body fluid or tissue suspected of harboring a tumor, such as alveolar, bronchiolar, or
20 respiratory epithelial cells obtained from a bronchial wash, nasopharyngeal aspirates, throat swabs or the like; (2) a metastasized neuroendocrine tumor, by biopsy, taken from tissue other than the adrenal gland (including cortex and medulla. Immuno-histochemical studies can be performed on
25 such cells using a monoclonal antibody specific for Dlk.

Diagnostic applications of these antibodies are exemplified, according to the present invention, by the use of a kit containing an anti-Dlk antibody, which undergoes a reaction with a biological sample to detect Dlk protein. Such
30 a reaction involves the binding of anti-Dlk antibody to Dlk antigen, under conditions permissive of binding. The observation of an antibody-antigen complex in a biological sample indicates a positive result. A kit of this sort could be used to detect the extent of expression of Dlk in a
35 particular biological sample from an individual, animal, or cell line.

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Such an immunodiagnostic kit can include anti-Dlk antibody and a receptacle for containing the antibody in a sterilized form. The kit can further include anti-isotype serum antibody which recognizes the anti-Dlk antibody (Fc portion) and which is conjugated to a label, such as an enzyme or fluorescent moiety.

In a preferred embodiment, a radiolabeled anti-Dlk antibody is provided. Such an antibody, preferably a monoclonal antibody, is administered to an animal or person for imaging purposes. After a suitable period of time for the administered antibodies to bind Dlk expressing cells, a gamma camera machine is applied to detect the presence of labeled antibodies within the organism. Such a procedure provides information as to where in the organism a primary or secondary Dlk-expressing neuroendocrine tumor is located.

A therapeutic application of anti-Dlk monoclonal antibodies includes administration of anti-Dlk immunotoxins. Conjugation of an anti-Dlk monoclonal antibody to a toxin, such as *Pseudomonas* exotoxin or other toxins commonly conjugated to an antibody by means of a conventional antibody-toxin linkage. Hertler et al., *J. Clin. Oncol.* 7(12): 1932 (1989), describe methodologies for creating an antibody-toxin linkage, and is incorporated by reference herein. Thus, the anti-Dlk monoclonal antibody-toxin conjugates described are administered to an individual to target and selectively kill Dlk-expressing cells present in neuroendocrine tumors.

Similarly, a kit is provided which contains anti-Dlk immunotoxins in a receptacle. A kit can include the anti-Dlk immunotoxins and a pharmaceutical excipient in a receptacle.

The present invention is further described with reference to the following, illustrative examples. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of the invention. Although any method and materials similar or equivalent to those described herein can be used in the practice of the invention, the preferred methods and materials have been described. Unless mentioned

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otherwise, the techniques employed or contemplated herein are standard methodologies known to the art. The materials, methods and examples are illustrative only and not limiting.

5 Example 1. IDENTIFICATION OF *dlk* POLYNUCLEOTIDE AND
POLYPEPTIDE MOLECULES

Identification of *dlk*

 In investigating molecules associated with the gastrin-releasing peptide (GRP) responsive phenotype, genes were
10 identified which both were: (1) expressed differentially between responsive murine Swiss and unresponsive murine Balb/c 3T3 fibroblasts, and (2) expressed in GRP-responsive human SCLC cell lines. The rationale for this approach was that gene products which correlated with a GRP-responsive phenotype
15 would be missing from Balb/c and unresponsive SCLC cell lines, but present in Swiss 3T3 fibroblasts and responsive SCLC cell lines.

 A differential cDNA library was constructed that was enriched for clones expressed in Swiss 3T3 but not in Balb/c
20 3T3 fibroblasts. The differential library of Swiss 3T3 compared with Balb/c 3T3 fibroblasts was constructed as explained in detail in Timblin et al., *Nucleic Acids Res.* 18: 1587 (1990). The RNA isolation, electrophoresis, northern blots, and hybridization techniques were performed as
25 described in Davis, et al., *BASIC METHODS IN MOLECULAR BIOLOGY*, Elsevier, New York, (1986). The nucleic acid probes were labelled with ³²P dCTP (Amersham, Arlington Heights, IL) by random priming as described in Ausubel et al., *CURRENT PROTOCOLS IN MOLECULAR BIOLOGY*, John Wiley and Sons, New York
30 3.5.9.-3.5.10 (1991).

 A partial length clone (150 nucleotides long) isolated from this differential library hybridized with a 1.6 kilobase mRNA which exhibited an expression pattern consistent with the two screening requirements. This partial length clone was
35 then used to screen a commercially available oligo dT-primed cDNA library of Swiss 3T3 fibroblasts in the λZAPII vector (Stratagene (La Jolla, CA)), to obtain a full length clone.

Screening procedures and plasmid rescue of positive λ ZAPII clones were performed following the manufacturer's (Stratagene) protocol, as described by Short et al., *Nuc. Acids Res.* 16: 7583 (1988). Several clones with inserts of approximately 1.6 kilobase pairs in length were obtained from this screening procedure.

DNA Sequencing

Rescued plasmids were sequenced with Sequenase (USB, Cleveland, OH) by the dideoxy chain termination method, according to the manufacturer's protocol described by Tabor et al., *J. Biol. Chem.* 214: 6447 (1989). Nucleotide sequence analysis defined an open reading frame of 1155 nucleotides, encoding a putative protein (Dlk) of 385 amino acids with a molecular weight of 41,320 daltons. This open reading frame was classified as coding by both Fickett's and Shepherd's methods. Fickett et al., *Nucleic Acids Res.* 10: 5303 (1982); Shepherd et al., *Meth. Enzymol.* 188: 180 (1990). The open reading frames were identified by software programs implementing these methods (PC/Gene software package, Intelligenetics Inc. (Mountain View, CA); A. Bairoch, Ph.D thesis, University of Geneva, (1990)).

In vitro Translation of Dlk Polypeptide

In vitro translation assays from mouse *dlk* mRNA were performed using a rabbit reticulocyte lysate system from NEN (Boston, MA), according to manufacturer's protocol, as described by Lockhard et al., *Biochem. Biophys. Res. Comm.* 37: 204 (1969).

The *dlk* mRNA was selected by hybridization of poly A+ RNA from Swiss 3T3 fibroblasts with denatured full-length *dlk* immobilized on nitrocellulose filters. (*dlk* mRNA was selected by hybridization of 2 μ g of poly A+ Swiss 3T3 RNA with 5 μ g of nitrocellulose-immobilized denatured *dlk*.) The bound RNA was eluted by boiling. Mouse *dlk* mRNA was also prepared *in vitro* using two different full length *dlk* cDNAs cloned in pGEM4Z (Promega). These three mRNAs were used as templates for *in vitro* translation.

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Labelled proteins were analyzed in a 12% polyacrylamide gel followed by fluorography. A protein band of approximately 42 kilodaltons was present in all three samples, in agreement with the predicted molecular weight of *Dlk* polypeptide.

5 A Comparison between mice and humans

Mouse and human *dlk* polynucleotide sequences are 86.2% identical and 90.1% similarity in their amino acid sequence. They share many potential sites of biological activity, including 6 EGF-like repeats (highly homologous to those found
10 in invertebrate neurogenic proteins) an integral transmembrane domain and a signal peptide domain.

The structural characteristics of *dlk* were analyzed with the program PC/Gene (Intelligenetics Inc. (Mountain View, CA), A. Bairoch, Ph.D thesis, University of Geneva (1990)). The
15 transmembrane domain was identified using the program RAOARGOS, implementing the method of Rao and Argos, *Biochim. Biophys. Acta* 869: 197 (1986). The signal peptide was analyzed with the program PSIGNAL, according to the method of Von Heijne, *Nucleic Acids Res.* 14: 4683 (1986).

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Example 2. A COMPARISON BETWEEN pPG2 and *dlk* GENE EXPRESSION IN MICE, & *dlk* GENE EXPRESSION IN HUMANS

In normal tissues of human, mouse and hamster origin, *dlk* expression was detected according to the present invention,
25 only in adrenal and placental tissue. Similarly, pG2 expression was known to be restricted to adrenal glands in normal human tissues.

The *dlk* mRNA was detected by Northern analysis in human and rat pheochromocytoma (PC12) cell lines. pG2 was
30 identified in pheochromocytoma cell lines by Helman et al., *PNAS USA* 84: 2336 (1987).

According to the present invention, *dlk* was detected in neuroblastoma (SK-N-SH) cells. pG2 expression in neuroblastoma cell lines was detected in differentiated cells,
35 but absent from undifferentiated neuroblastoma cell lines. Cooper et al., *Cell Growth and Diff.* 1: 149 (1989).

In addition, other cells which express *dlk* identified by the present invention include certain SCLC cell lines. Also, murine Swiss 3T3 fibroblasts were found to express *dlk* using a human *dlk* probe under high stringency conditions. Balb/c 3T3 fibroblasts were found to express *dlk* using a human *dlk* probe under high stringency conditions. Balb/c 3T3 fibroblasts RNA were negative for *dlk* expression under these conditions.

To explore the relationship between mouse *dlk* and human pG2, cDNA clones were isolated from a λ gt10 human adrenal gland library (Clontech, Palo Alto, CA) using mouse *dlk* as a hybridization probe. Even under low stringency conditions, no clones were isolated which encoded proteins having structural characteristics similar to those reported for pG2. The cDNA inserts from positive λ clones were subcloned into pGEM4Z (Promega, Madison WI) and sequenced according to the method of Example 1. Polynucleotide sequence data from several full-length clones indicated that these cDNAs showed a 82.1% sequence identity with mouse *dlk* and encoded the human homolog of the mouse *dlk* protein (Fig. 1).

Dlk's structural characterization is very different from that predicted for pG2 protein (Helman et al., *supra*. (1987). the former protein consists of a 286 amino acid sequence (about 30 kDa), contains no EGF-like repeats and no signal peptide or transmembrane domains. This was so, despite a finding of an 81.2% nucleotide sequence identity of *dlk* with pG2 is identified correctly as the *dlk* polynucleotide molecule shown in Figure 1.

Example 3. *dlk*/Dlk HOMOLOGY WITH OTHER GENES & PROTEINS

The *dlk* nucleic acid sequence shows a high degree of homology with the EGF-like neurogenic genes of *Drosophila*, which are involved in the decisions taken by the cells of the embryonal ectoderm to differentiate into epidermal or neuronal cells. Genes which were found to have highest homology to Dlk include: Delta, Notch and Serrate of *D. melanogaster*, lin-12 and glp1 of *C. elegans*, and uEGF1 of the sea urchin. Although

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the degree of homology varied between the individual proteins and Dlk, regions of maximum homology exhibited up to 33% amino acid identity, which rose to around 75%, with allowance for conservative amino acid substitutions.

5 Figure 4 shows the alignment of mouse or human *dlk* EGF-like repetitive sequences with consensus sequences of EGF sequence repeats of several proteins. The alignment of the EGF-like repeats was done using the program CLUSTAL, described by Higgins et al., *Gene* 73: 237 (1988). The sites of
10 potential biological importance were analyzed with the program PROSITE. Residues highly conserved among homeotic genes also are conserved in *dlk*. This finding confirms that *dlk* is a member of the family of EGF-like homeotic genes. The amino acid sequence and structure of the EGF-like repeats, as well
15 as the overall structure of *dlk*, are more related to the invertebrate homeotic genes than to other vertebrate non-homeotic EGF-like proteins, such as EGF-precursor, TGF α , the α , β 1 and β 2 chains of laminin, coagulation factors, or complement proteins, previously thought to be the mammalian
20 counterparts of the invertebrate homeotic genes.

 The *dlk* gene was detected in species ranging from birds to human, including: yeast, *Drosophila*, *Xenopus*, mouse, rat, rabbit, chicken, dog, cow, monkey and human. However, despite the structural homology with invertebrate proteins, the *dlk* gene is absent from invertebrates and lower vertebrates.
25

 The program PCOMPARE, described by Needleman et al., *Mol. Biol.* 48: 443 (1970), included in PC/Gene was used for the analysis of homology. In this method, the optimal alignment score between two proteins were compared with the statistical
30 distribution of 100 random alignments. An alignment score of greater than 5 positive standard deviations from the mean random alignment distribution was considered significant, particularly when no functional or structural relationship between the proteins compared is known. Representative
35 alignment scores were determined: Delta, 20.2; Serrate, 19.7; TAN-1, 16.2; Notch, 14.6; Xotch, 13.6; *Drosophila* Laminin β 2,

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6.3; mouse Laminin $\beta 2$, 4.1; human coagulation factor XII, 2.8; and human EGF precursor, 0.6.

Example 4. *dlk* EXPRESSION: NORTHERN BLOT mRNA ANALYSIS

5 Expression of *dlk* was detected by Northern analysis in
SCLC lines NCI-H510, NCI-H69 and NCI-N592; in human
neuroblastoma line SK-N-SH, and in the rat pheochromocytoma
PC-12 cell line. Twenty μ g of total RNA or 2 μ g of poly A+
10 were separated in a 1% agarose gel and then blotted to a
nitrocellulose filter (described in Example 1).

 A 1.6 Kb band corresponding to *dlk* was observed only in
the RNA samples from the SCLC cell lines NCI-N592, NCI-H69 and
NCI-H510, and in Swiss 3T3 fibroblasts. Mouse Swiss 3T3
15 fibroblast RNA also showed a high degree of expression of *dlk*,
even when the hybridization was performed under high
stringency conditions using human *dlk* as a probe. Similar
results were obtained using mouse *dlk* as a probe. Balb/c 3T3
fibroblast RNA was negative for *dlk* expression under these
conditions. Ewing's sarcoma cell lines SK-ES-1, A4573 and
20 TC106 did not express *dlk*.

 In normal tissues of mouse, hamster, and human origin *dlk*
expression was detected exclusively in the adrenal gland.

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What Is Claimed Is:

1. An isolated Dlk polypeptide consisting essentially of the amino acid sequence shown in Figure 1B.

5 2. An isolated polynucleotide molecule which encodes a human Dlk polypeptide according to Claim 1.

3. An isolated polynucleotide molecule consisting essentially of the polynucleotide sequence shown in Figure 2.

10 4. An isolated polynucleotide molecule which encodes a murine Dlk polypeptide, wherein said Dlk polypeptide comprises the amino acid sequence shown in Figure 1A.

5. A method for detecting a tumor which expresses *dlk*, comprising the steps of

15 (a) contacting a sample suspected of being tumorigenic with *dlk* polynucleotide molecule, under conditions permissive of hybridization between *dlk* polynucleotide molecule and said sample and

(b) detecting the presence of hybridization between said polynucleotide molecule and said sample.

20 6. A method according to Claim 5, wherein said *dlk* polynucleotide molecule is an isolated polynucleotide molecule according to Claim 3.

7. A method according to Claim 5 for detecting a tumor identified as small cell lung carcinoma, wherein, in step (a), said sample comprises bronchial epithelial cells.

Figure 2

	3	9	15	21	27	33	39	45								
1	TCT	AAA	GGA	GGT	GGA	GAG	CGC	ACC	GCA	GCC	CGG	TGC	AGC	CCG	GTG	
46	CAG	CCC	TGG	CTT	TCC	CCT	CGC	TGC	GGC	CCG	TGC	CCC	CTT	TCG	CGT	
91	CCG	CAA	CCA	GAA	GCC	CAG	TGC	GGC	GCC	AGG	AGC	CGG	ACC	CGC	GCC	
136	CGC	ACC	GCT	CCC	GGG	ACC	GCG	ACC	CCG	GCC	GCC	CAG	AGA	TGA	CCG	
181	CGA	CCG	AAG	CCC	TCC	TGC	GCG	TCC	TCT	TGC	TCC	TGC	TGG	CTT	TCG	
226	GCC	ACA	GCA	CCT	ATG	GGG	CTG	AAT	GCT	TCC	CGG	CCT	GCA	ACC	CCC	
271	AAA	ATG	GAT	TCT	GCG	AGG	ATG	ACA	ATG	TTT	GCA	GGT	GCC	AGC	CTG	
316	GCT	GGC	AGG	GTC	CCC	TTT	GTG	ACC	AGT	GCG	TGA	CCT	CTC	CCG	GCT	
361	GCC	TTC	ACG	GAC	TCT	GTG	GAG	AAC	CCG	GGC	AGT	GCA	TTT	GCA	CCG	
406	ACG	GCT	GGG	ACG	GGG	AGC	TCT	GTG	ATA	GAG	ATG	TTC	GGG	CCT	GCT	
451	CCT	CGG	CCC	CCT	GTG	CCA	ACA	ACG	GGA	CCT	GCG	TGA	GCC	TGG	ACG	
496	ATG	GCC	TCT	ATG	AAT	GCT	GCA	CCC	CTG	CCC	CCG	GGT	ACT	CGG	GAA	AGG
541	ACT	GCC	AGA	AAA	AGG	ACG	GGC	CCT	GTG	TGA	TCA	ACG	GCT	CCC	CCT	
586	GCC	AGC	ACG	GAG	GCA	CCT	GCG	TGG	ATG	ATG	AGG	GCC	GGG	CCT	CCC	
631	ATG	CCT	CCT	GCC	TGT	GCC	CCC	CTG	GCT	TCT	CAG	GCA	ATT	TCT	GCG	
676	AGA	TCG	TGG	CCA	ACA	GCT	GCA	CCC	CCA	ACC	CAT	GCG	AGA	ACG	ACG	
721	GCG	TCT	GCA	CTG	ACA	TTG	GGG	GCG	ACT	TCC	GCT	GCC	GGT	GCC	CAG	
766	CCG	GCT	TCA	TCG	ACA	AGA	CCT	GCA	GCC	GCC	CGG	TGA	CCA	ACT	GCG	
811	CCA	GCA	GCC	CGT	GCC	AGA	ACG	GGG	GCA	CCT	GCC	TGC	AGC	ACA	CCC	
856	AGG	TGA	GCT	ACG	AGT	GTC	TGT	GCA	AGC	CCG	AGT	TCA	CAG	GTC	TCA	
901	CCT	GTG	TCA	AGA	AGC	GCG	CGC	TGA	GCC	CCC	AGC	AGG	TCA	CCC	GTC	
946	TGC	CCA	GCG	GCT	ATG	GGC	TGG	CCT	ACC	GCC	TGA	CCC	CTG	GGG	TGC	
991	ACG	AGC	TGC	CGG	TGC	AGC	AGC	CGG	AGC	ACC	GCA	TCC	TGA	AGG	TGT	
1036	CCA	TGA	AAG	AGC	TCA	ACA	AGA	AAA	CCC	CTC	TCC	TCA	CCG	AGG	GCC	
1081	AGG	CCA	TCT	GCT	TCA	CCA	TCC	TGG	GCG	TGC	TCA	CCA	GCC	TGG	TGG	
1126	TGC	TGG	GCA	CTG	TGG	GTA	TCG	TCT	TCC	TCA	ACA	AGT	GCG	AGA	CCT	
1171	GGG	TGT	CCA	ACC	TGC	GCT	ACA	ACC	ACA	TGC	TGC	GGA	AGA	AGA	AGA	
1216	ACC	TGC	TGC	TTC	AGT	ACA	ACA	GCG	GGG	AGG	ACC	TGG	CCG	TCA	ACA	
1261	TCA	TCT	TCC	CCG	AGA	AGA	TCG	ACA	TGA	CCA	CCT	TCA	GCA	AGG	AGG	
1306	CCG	GCG	ACG	AGG	AGA	TCT	AAG	CAG	CGT	TCC	CAC	AGC	CCC	CTC	TAG	
1351	ATT	CTT	GGA	GTT	CCG	CAG	AGC	TTA	CTA	TAC	GCG	GTC	TGT	CCT	AAT	
1396	CTT	TGT	GGT	GTT	CGC	TAT	CTC	TTG	TGT	CAA	ATC	TGG	TGA	ACG	CTA	
1441	CGC	TTA	CAT	ATA	TTG	TCT	TTG	TGC	TGC	TGT	GTG	ACA	AAC	GCA	ATG	
1486	CAA	AAA	CAA	TCC	TCT	TTC	TCT	CTC	TTA	ATG	CAT	GAT	ACA	GAA	TAA	
1531	TAA	TAA	GAA	TTT	CAT	CTT	TAA	ATG	AG							

Total number of bases is: 1556.

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Figure 3

	3	9	15	21	27	33	39	45
1	GGT	GCA	ACC	CTA	GCT	TTC	TTC	CCG
46	GTG	GTC	CGC	AAC	CAG	AAG	CCC	AGC
91	GCA	CCG	CCT	CCG	CTC	CCC	GGA	CCG
136	GAT	CGC	GAC	CGG	AGC	CCT	CCT	GCG
181	TTT	CGG	CCA	CAG	CAC	CTA	TGG	GGC
226	CCC	CCA	GTA	TGG	ATT	CTG	CGA	GGC
271	TGT	TGG	CTG	GGA	GGG	TCC	CCT	CTG
316	TGG	CTG	TGT	CAA	TGG	AGT	CTG	CAA
361	CAA	GGA	TGG	CTG	GGA	CGG	GAA	ATT
406	TTG	CAC	CTC	AAC	CCC	CTG	CGC	CAA
451	GGA	GAA	AGG	CCA	GTA	CGA	ATG	CTC
496	AAA	GGA	CTG	CCA	GCA	CAA	GGC	TGG
541	TCC	CTG	CCA	GCA	CGG	AGG	CGC	CTG
586	CTC	GCA	TGC	TTC	CTG	CCT	GTG	CCC
631	CTG	TGA	GAT	CGT	AGC	CGC	AAC	CAA
676	CGA	GAA	CGA	TGG	CGT	CTG	CAC	CGA
721	CCG	CTG	CCC	AGC	TGG	ATT	CGT	CGA
766	GAG	CAA	CTG	CGC	CAG	TGG	CCC	GTG
811	CCA	GCA	CAC	CCA	GGT	GAG	CTT	CGA
856	CAT	GGG	TCC	CAC	GTG	CGC	GAA	GAA
901	GGT	CAC	CCA	CCT	GCC	CAG	CGG	CTA
946	CCC	CGG	GGT	GCA	CGA	GCT	GCC	TGT
991	CCT	GAA	GGT	GTC	CAT	GAA	AGA	GCT
1036	CAC	GGA	GGG	ACA	GGC	CAT	CTG	CTT
1081	CAG	CCT	GGT	GGT	GCT	GGG	CAC	CGT
1126	GTG	CGA	AAC	CTG	GGT	GTC	CAA	CCT
1171	CAA	GAA	GAA	GAA	CCT	CCT	GTT	GCA
1216	GGC	GGT	CAA	TAT	CAT	CTT	CCC	CGA
1261	CAA	CAA	GGA	GGC	TGG	TGA	TGA	GGA
1306	CCC	CAC	TCC	CAG	GCC	CTT	CAC	CCC
1351	TAT	TAC	CGG	GTT	CCT	TTA	GAG	CTC
1396	TGG	TGG	AGT	TTG	CTC	TAT	TGT	GTG
1441	TAC	ATA	TAT	TGT	CTT	GTG	TTG	CTG
1486	CTA	AGA	ACC	CCT	TCC	TCC	CTA	TTA
1531	TAA	TAA	GAA	TTT	CAT	CTC	TAA	ATG

Total number of bases is: 1573.

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Consensus

dlk	C	PC	NGG	C	D	Y	C	C	PGF	G	C	
Delta	C	PC	NGGTC			Y	C	C	GF	G	C	
Serrate	C	PC	NGGTC	D		F	C	C	G	G	C	
TAN-1	C	PC	NGGTC	D		Y	C	C	GF	G	C	
Notch	C	S	PC	NGGTC	D	Y	C	C	GF	G	C	
Xotch	C	PC	NGGTC	D		Y	C	C	GF	G	C	
Lin-12	CL		C	N	G	CI	Y	C	C	GY	G	C

EGF mouse CPSSYDGYCLNGGVCMHIESDLSYT CNCVIGYSGDRC

FIGURE 4

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